Title: Development and evaluation of novel real-time RT-PCR assays with locked nucleic acid probes targeting the leader sequences of human pathogenic coronaviruses

Running title: Novel RT-PCR assays targeting CoV leader sequences

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ABSTRACT

Based on small-RNA-Seq analysis data, we developed highly sensitive and specific real-time RT-PCR assays with locked nucleic acid probes targeting the abundantly expressed leader sequences of MERS-CoV and other human coronaviruses. Analytical and clinical evaluations showed their non-inferiority to a commercial multiplex PCR test for the detection of these coronaviruses.

Coronaviruses (CoVs) have repeatedly crossed species barriers and some have emerged as important human pathogens (1,2). HCoV-229E, HCoV-OC43, HCoV-NL63, and HCoV-HKU1 predominantly cause mild upper respiratory tract infections, while severe acute respiratory syndrome CoV (SARS-CoV) and Middle East respiratory syndrome CoV (MERS-CoV) frequently cause severe pneumonia with extrapulmonary manifestations (3-6). Highly sensitive and specific laboratory diagnostic tests are essential for the control of emerging CoV outbreaks (7). The gold standard of laboratory diagnosis of CoV infection is the isolation of infectious virus from respiratory tract and/or other clinical specimens. However, most CoVs are either difficult or dangerous to culture in cell lines (8,9). The need of convalescent samples and potential false-positive results due to cross-reactivity with other CoVs limit the use of serum antibody detection assays in the acute setting (10). The overall sensitivity of antigen detection assays is inferior to that of molecular assays such as reverse transcription (RT)-PCR (11,12). With the increasing availability of molecular diagnostic facilities and expertise in clinical microbiology laboratories worldwide, RT-PCR has become the test of choice for diagnosing CoV infections (7,13-15).

Traditionally, the preferred targets of RT-PCR assays are genes that are conserved and/or abundantly expressed from the viral genome (16). For CoVs, the most commonly employed targets include the structural nucleocapsid (N) and spike (S) genes, and the non-structural RNA-dependent RNA polymerase (RdRp) and replicase ORF1a/b genes (4,7). Recently, other unique non-coding genome regions not present in related CoVs have also been utilized to develop RT-PCR for the emerging MERS-CoV (7,13-15). The World Health Organization (WHO) recommends using the upE assay (regions upstream of the envelope [E] gene) for laboratory screening of suspected MERS cases, followed by confirmation with either the ORF1a or ORF1b assays (7). Notably, a number of single nucleotide mismatches at different positions included in

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the upE assay forward primer and probe have been detected in recent strains of MERS-CoV and may affect the sensitivity of this assay (17). We hypothesize that additional gene targets may be suitable for RT-PCR design for CoVs and would increase the options of molecular diagnosis for circulating and emerging CoV infections. In this study, we designed and evaluated novel real-time RT-PCR assays with locked nucleic acid (LNA) probes for clinically important CoVs based on the identification of the abundantly expressed leader sequence in the 5'-untranslated region (UTR) in small-RNA-Seq data analysis.

We included MERS-CoV (strain HCoV-EMC/2012, passage 8, provided by Ron Foucheir, Erasmus Medical Center), HCoV-229E, HCoV-OC43, HCoV-NL63, and HCoV-HKU1 in the study. SARS-CoV was not included as there has not been any human case since 2005. The MERS-CoV isolate was amplified by one additional passage in Vero cells to make working stocks of the virus (5.62×10^5 50% tissue culture infective doses [$TCID_{50}$]/ml) as previously described (18). All experimental protocols involving live MERS-CoV followed the approved standard operating procedures of the biosafety level 3 facility at Department of Microbiology, The University of Hong Kong, as previously described (19). High-titer stocks of HCoV-229E, HCoV-OC43, and other respiratory viruses were prepared and their $TCID_{50}$ values were determined using standard methods and as previously described (20,21-23). Attempts to culture HCoV-NL63 and HCoV-HKU1 were unsuccessful because of their difficulty to grow in cell lines available in our laboratories. Virus-positive clinical specimens (n=14) and laboratory strains (n=13) used for evaluating the novel assays' cross-reactivities with other respiratory viruses were obtained from archived nasopharyngeal aspirates at the clinical microbiology laboratory at Queen Mary Hospital, Hong Kong. Total nucleic acid extractions of clinical specimens and laboratory cell culture with virus strains were performed on 200 µl of sample

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using EZ1 virus Mini Kit v2.0 (QIAgen) according to the manufacturer's instructions. The elution volume was 60 µl. Extracts were stored at -70°C or below until use. Total nucleic acid extracts of ResPlex-II-HCoV-positive (n=49) and -negative (n=180) respiratory clinical specimens prepared by using the QIAamp MinElute Virus Spin Kit were provided by the Hong Kong Sanatorium and Hospital. A total of 229 fresh or frozen nasopharyngeal aspirates (NPAs) collected between 1 January 2012 and 31 October 2014 from 229 pediatric and adult patients, including 128 males and 101 females, aged 1 to 97 years, who were managed in Queen Mary Hospital and Hong Kong Sanatorium and Hospital for upper and/or lower respiratory symptoms were included in the study.

The most abundantly expressed sequence in the MERS-CoV genome was determined by small-RNA-Seq data analysis (Supplementary Information). Approximately 2.6% of the trimmed reads could be mapped onto the MERS-CoV genome. Among the mapped sequences, the mapping analysis revealed that most of these small RNA sequence reads, accounting for >6,000 sequences (6.3%), matched the 67-nucleotide leader sequence at the 5' terminus of the genome (Fig. 1). In contrast, the other peaks at the ORF1a, S, and N gene regions accounted for <3.0% of the mapped small RNA sequence reads. Our mapping analysis also showed that the percentages of mapped small RNA sequence reads at the gene regions targeted by the previously described upE, ORF1a, ORF1b, N2, N3, NSeq, and RdRpSeq assays, which had longer sequences than the 67-nucleotide MERS-CoV leader sequence, were only 0.2%, 0.1%, <0.1%, 0.3%, 0.1%, 2.8%, and 0.2% respectively (13,14,24). HCoV-229E, HCoV-OC43, HCoV-NL63, and HCoV-HKU1 similarly possess 70 to 72-nucleotide leader sequences at the same region in their respective genomes (Fig. 1) (25-28). Although leader sequences of around 60 to 90 nucleotides in length are found at the 5'-UTR upstream to the transcription regulatory sequence in the genomes and at

the subgenomic RNAs of all CoVs, the function of these leader sequences remains poorly understood (29-31). In view of the abundance of the leader sequences and since infected cells are known to contain large amounts of viral subgenomic RNA at which the leader sequences are abundantly found (32,33), we hypothesized that the leader sequence might be a valuable diagnostic target not only for MERS-CoV but also for other currently circulating HCoVs (Fig. 1).

To overcome the relatively short length of the leader sequences, we employed LNA probes (Exiqon, Copenhagen, Denmark) to develop novel real-time RT-PCR assays for these human pathogenic CoVs. LNA is a nucleic acid analogue with an extra bridge connecting the 2' oxygen and 4' carbon that has exceptionally high hybridization affinity towards complementary DNA and RNA and efficient mismatch discrimination (34). These properties are associated with an increased melting temperature of the oligonucleotides, which allows the application of shorter probes when LNA rather than DNA nucleotides are used in the nucleic acid amplification assays (34). In recent years, LNA probes have been increasingly used in the design of real-time PCR assays for other respiratory infections such as those caused by avian influenza A/H5N1 virus, rhinovirus, enteroviruses, respiratory syncytial virus, and *Mycoplasma pneumoniae* (35-38). Using LNA probes, we developed five novel real-time RT-PCR assays (named MERS-CoV-LS, HCoV-229E-LS, HCoV-OC43-LS, HCoV-NL63-LS, and HCoV-HKU1-LS) targeting the short leader sequences of these CoVs (Table 1).

The analytical sensitivities and specificities of the assays were excellent. The limits of detection with *in vitro* RNA transcripts for MERS-CoV-LS, HCoV-229E-LS, and HCoV-OC43-LS were 10 RNA copies/reaction and those for HCoV-NL63-LS and HCoV-HKU1-LS were 5 RNA copies/reaction. Linear amplification was achieved over an 8-log dynamic range, from 10^1-

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10^8 RNA copies/reaction for all five assays, with calculated linear correlation coefficients (R^2) of 0.99-1.00 and amplification efficiency values of 1.93-2.27. The limits of detection with viral RNA were approximately 5.62×10^{-2} $TCID_{50}$/ml, 5.00×10^{-2} $TCID_{50}$/ml, and 3.16×10^{-3} $TCID_{50}$/ml for MERS-CoV-LS, HCoV-229E-LS, and HCoV-OC43-LS respectively (Supplementary Tables 1 and 2). The limit of detection for the MERS-CoV-LS assay was about one log $TCID_{50}$/ml higher than that for the MERS-CoV-upE assay in parallel runs and was comparable with those for the other assays currently recommended for screening and/or confirmation of MERS by the WHO, including the ORF1a, ORF1b, RdRpSeq, and NSeq assays (Supplementary Table 1) (7,14). Comparatively, the ORF1b assay for MERS-CoV has the least optimal limit of detection of 64 RNA copies/reaction (13,14). Our assays showed no cross-reactivity among the individual CoVs and with other common respiratory viruses including adenovirus, influenza A and B viruses, parainfluenza virus types 1 to 4, rhinovirus, respiratory syncytial virus, and human metapneumovirus (Supplementary Table 3).

Additionally, we assessed the diagnostic performance of our assays and compared it with ResPlex-II in in-use evaluation using 229 NPAs. ResPlex-II is a commercially available multiplex PCR assay which detects 18 respiratory viruses including HCoV-229E, HCoV-OC43, HCoV-NL63, and HCoV-HKU1 in a single run. It is commonly employed for laboratory diagnosis of viral respiratory tract infections in many clinical laboratories worldwide (39,40). Forty-nine NPAs which were tested positive for HCoVs by ResPlex-II and another 180 NPAs which were tested negative for respiratory viruses by ResPlex-II (Table 2) were tested in an operator-blinded manner. Our assays tested positive for the corresponding HCoVs with viral loads of 1.37×10^{1}-3.86×10^{8} RNA copies/reaction in all 49 (100%) ResPlex-II-HCoV-positive NPAs (Table 2) (p = 1.00; Fisher's exact test). Moreover, our assays detected HCoVs in an

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additional 4/180 (2.2%) NPAs which were initially tested negative by ResPlex-II, probably because of the low viral loads of 2.29×10^{1}-2.40×10^{2} RNA copies/reaction (p = 0.12; Fisher's exact test). Sequencing analysis and two-step confirmatory real-time RT-PCR assays using specific primers targeting the N gene of HCoV-OC43 and HCoV-NL63 (Supplementary Information) confirmed that the results of these four ResPlex-II-HCoV-negative specimens concurred with their CoV real-time RT-PCR assay results (two were positive for HCoV-OC43 and two were positive for HCoV-NL63). Overall, these results suggest that our assays are highly sensitive and specific, and not inferior to ResPlex-II for the detection of HCoVs *in vitro* and in clinical samples. It is important to note that while ResPlex-II and other multiplex PCR assays have the advantage of being able to detect multiple viruses simultaneously, the sensitivity may be <50% and inferior to monoplex PCR assays for HCoVs and other respiratory viruses such as influenza A viruses (39,41). This relatively poorer sensitivity would especially limit the application of these multiplex PCR assays for the detection of future emerging CoVs and avian influenza A viruses which are potential pandemic agents that have significant public health impact if a case was misdiagnosed.

Our study has demonstrated the previously unknown diagnostic value of the CoV leader sequence and the usefulness of small-RNA-Seq data analysis in the selection of optimal gene targets for the development of molecular diagnostic assays. The application of LNA probes allowed the use of relatively short sequences such as the leader sequence of CoV genomes as a diagnostic target in RT-PCR assays. The same approach may be applied to identify and design real-time RT-PCR assays for other emerging viruses including novel CoVs that are likely to emerge in the future, once their genomic data become available. As for any other gene targets used in RT-PCR assays, particular attention should be paid to the presence of polymorphisms in

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the leader sequences, which may affect the sensitivity of the assays. The novel CoV real-time RT-PCR assays with LNA probes described in the present study should be further evaluated in large-scale in-field evaluations. Development of these assays into multiplex assays with comparable sensitivity and specificity and additional detection of other novel or re-emerging CoVs may further enhance their clinical utility.

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182 **Ethical approval.** The study was approved by the Institutional Review Board of The University
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184

185 **Conflict of interests:** None.

186

FIGURE LEGENDS

Fig. 1 Schematic diagram of the MERS-CoV genome, with the leader sequence at the 5'-untranslated region enlarged to illustrate the abundance of the small RNA sequences. The percentages of mapped small RNA sequence reads at the leader sequence, ORF1a, S, and N gene regions are quantified and shown. Leader sequences of 70 to 72 nucleotides in length are also present in other human coronaviruses (HCoV-229E, HCoV-OC43, HCoV-NL63, and HCoV-HKU1).

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TABLE 1 Primer and probe sequences of CoV real-time RT-PCR assays with LNA probes in the present study[a]

Assay	Genome target	Genome location	Primer/probe	Sequence (5' to 3')	GenBank accession no.
MERS-CoV-LS	Leader sequence	14-32	Forward	AGCTTGGCTATCTCACTTC	JX869059.2
		47-69	Reverse	AGTTCGTTAAAATCAAAGTTCTG	
		34-47	Probe	C+CT+CGT+T+CT+CT+TGC	
HCoV-229E-LS	Leader sequence	20-41	Forward	CTACAGATAGAAAAGTTGCTTT	NC_002645.1
		57-75	Reverse	ggTCGTTTAGTTGAGAAAAGT	
		44-59	Probe	AGACT+T+TG+TG+TCT+A+CT	
HCoV-OC43-LS	Leader sequence	17-28	Forward	aaaCGTGCGTGCATC	NC_005147.1
		43-66	Reverse	AGATTACAAAAAGATCTAACAAGA	
		32-48	Probe	C+TTCA+CTG+ATCT+C+T+TGT	
HCoV-NL63-LS	Leader sequence	23-46	Forward	ggAGATAGAGAATTTTCTTATTTAGA	NC_005831.2
		60-77	Reverse	ggTTTCGTTTAGTTGAGAAG	
		50-66	Probe	TGTGT+C+TAC+T+C+TTCT+CA	
HCoV-HKU1-LS	Leader sequence	21-37	Forward	CGTACCGTCTATCAGCT	NC_006577.2
		48-71	Reverse	GTTTAGATTTAATGAGATCTGACA	
		39-52	Probe	ACGA+T+CT+C+TTG+T+CA	

Abbreviations: HCoV, human coronavirus; MERS-CoV, Middle East respiratory syndrome coronavirus; UTR, untranslated region.

[a] Probes were labeled at the 5' end with the reporter molecule 6-carboxyfluorescein (6-FAM) and at the 3' end with Iowa Black FQ (Integrated DNA Technologies, Inc). Lowercase letters represent the additional bases added which is not from the original genome sequence. The letters following "+" represent LNA bases which are modified with an extra bridge connecting the 2' oxygen and 4' carbon. The bridge "locks" the ribose in the 3'-endo (North) conformations and significantly increases the hybridization properties of the probe.

203 **TABLE 2** Comparison between CoV real-time RT-PCR assays with LNA probes and ResPlex-II for the detection of CoVs in
204 nasopharyngeal aspirates
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	Resplex-II-HCoV-positive NPAs[a] (n = 49)				Resplex-II-HCoV-negative NPAs[a] (n = 180)
Assay	**HCoV-229E**	**HCoV-OC43**	**HCoV-NL63**	**HCoV-HKU1**	
MERS-CoV-LS	0/1	0/17	0/27	0/4	0/180
HCoV-229E-LS	1/1 (1.64×10^4)	0/17	0/27	0/4	0/180
HCoV-OC43-LS	0/1	17/17 (1.37×10^1 - 3.86×10^8)	0/27	0/4	2/180 (1.84×10^2 - 2.40×10^2)
HCoV-NL63-LS	0/1	0/17	27/27 (9.20×10^2 - 3.47×10^7)	0/4	2/180 (2.29×10^1 - 9.34×10^1)
HCoV-HKU1-LS	0/1	0/17	0/27	4/4 (1.94×10^3 - 4.42×10^5)	0/180

206 Abbreviations: NPAs, nasopharyngeal aspirates

207 [a] Values in brackets represent the ranges of quantitative results of positive samples in RNA copies/reaction.

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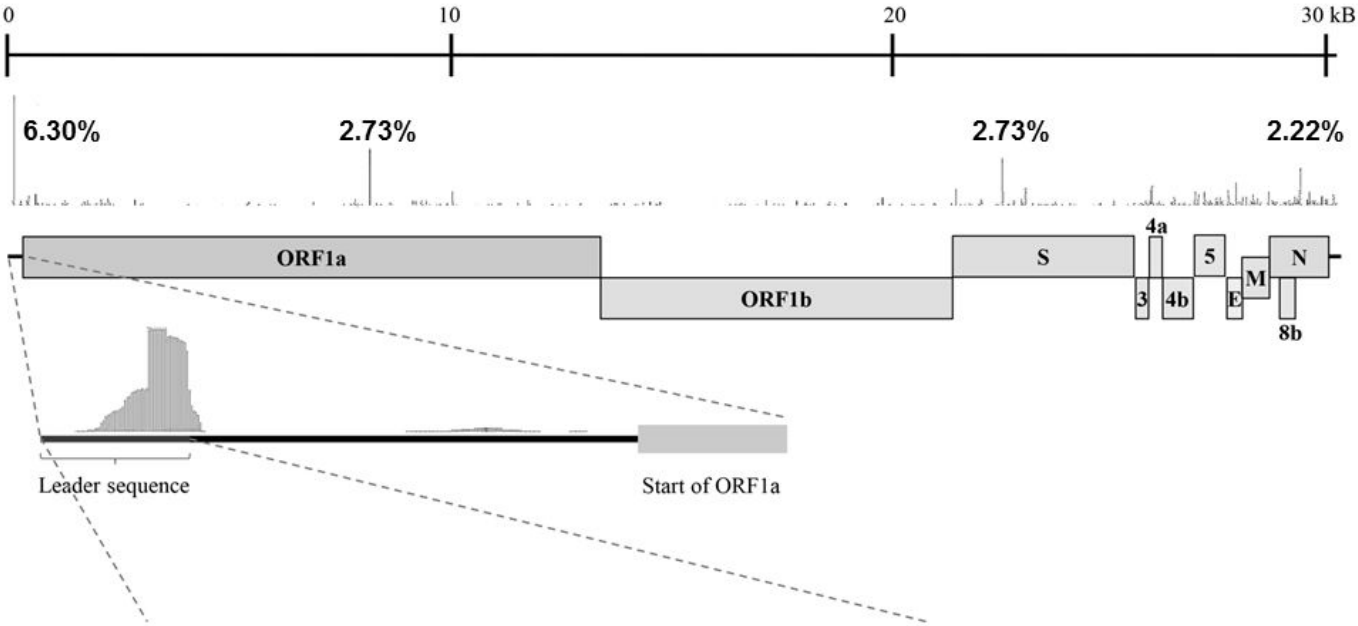


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MERS-CoV    GAUUUAAGUGAAUAGCUUGGCUAUCUCACUUCCCCUCGUUCUCUUGCAGAACUUUGAUUUUAACGAA
HCoV-229E   ACUUAAGUACCUUAUCUAUCUACAGAUAGAAAAGUUGCUUUUUAGACUUUGUGUCUACUUUUCUCAACUAA
HCoV-OC43   GAUUGUGAGCGAUUUGCGUGCGUGCAUCCCGCUUCACUGAUCUCUUGUUAGAUCUUUUUGUAAUCUAAAC
HCoV-NL63   CUUAAAGAAUUUUUCUAUCUAUAGAUAGAGAAUUUUCUUAUUUAGACUUUGUGUCUACUCUUCUCAACUAAA
HCoV-HKU1   GAGUUUGAGCGAUUGACGUUCGUACCGUCUAUCAGCUUACGAUCUCUUGUCAGAUCUCAUUAAAUCUAAAC
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